Sanglifehrins A, B, C and D, Novel Cyclophilin-binding Compounds Isolated from *Streptomyces* sp. A92-308110

I. Taxonomy, Fermentation, Isolation and Biological Activity

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A novel class of macrolides for which the name sanglifehrins is proposed, has been discovered from actinomycete strains based on their high affinity binding for cyclophilin A (CypA), an immunophilin originally identified as a cytosolic protein binding cyclosporin A (CsA). The sanglifehrins were produced by *Streptomyces* sp. A92-308110. They were isolated and purified by extraction and several chromatographic, activity-guided steps. Sanglifehrins A and B exhibit a 10~20 fold higher affinity for CypA than CsA, whereas the affinity of sanglifehrins C and D for CypA is comparable to that of CsA. Sanglifehrins exhibit a lower immunosuppressive activity than CsA when tested in the mixed lymphocyte reaction. Their *in vitro* activity indicates that they belong to a novel class of immunosuppressants.

Cyclosporin A (CsA), an undecapeptide produced by various fungi[1], exerts its immunosuppressive effects by binding to the intracellular binding protein cyclophilin A (CypA)[2]. Although this binding is required, it is not sufficient for the immunosuppressive activity. The CsA-CypA complex binds to another protein, the serine-threonine phosphatase calcineurin, whose enzymatic activity is inhibited[3,4]. This leads to the inhibition of T cell activation by preventing transcription of early T cell genes including genes encoding lymphokines like interleukin-2, the main growth factor for T cells[5].

To identify compounds which might potentially interfere with other signalling pathways not involving calcineurin and which thereby might exert novel biological effects, a screening for novel cyclophilin-binding entities was performed with methanolic extracts of actinomycete broths. This approach was stimulated by findings related to two other immunosuppressive drugs, namely FK506 and rapamycin. Both drugs bind to the same intracellular binding protein FKBP[6]. However, the corresponding drug-FKBP complexes interact with two different effector molecules: the FK506-FKBP complex binds to calcineurin as does the CsA-CypA complex. Consequently, FK506 inhibits T cell activation *via* the same signalling pathway as CsA does[7]. In contrast, the rapamycin-FKBP complex binds to a different protein, *i.e.* mTOR, which is involved in growth factor mediated intracellular signal-transduction pathways[8]. Accordingly, rapamycin has a different activity profile, inhibiting the clonal expansion of T cells at a later stage. The effect of rapamycin is not restricted to T cells; in general, rapamycin inhibits the proliferation of cells in response to growth factors[9].

Screening for microbial broth extracts blocking the CsA-CypA interaction was performed with a competitive ELISA . Among more than 12,000 actinomycete extracts tested (4,000 strains from various origin in three media) three showed activity in this binding assay. One strain produced the metabolites cymbimycin A and B[10], and one of the others, S92-308110A of the genus *Streptomyces* sp., lead to the discovery of the sanglifehrins.

Materials and Methods

Characterization of the Producing Strain

The morphology of the strain was ascertained by light microscopy and scanning electron microscopy. Growth characteristics and carbohydrate utilization were determined by the methods of the International *Streptomyces* Project (ISP)[11]. The analysis of diaminopimelic acid was performed on the hydrolysate of cells grown on Bennett's agar medium.[12] The analysis of the fatty acids and the whole-cell sugars was determined by gas chromatography[13].

Materials

For the production of sanglifehrins the seed medium was composed of glucose 1%, soluble starch 2%, yeast extract 0.5% (Gistex, Gist Brocades), *NZ*-amine Type A (Sheffield) 0.5%, $CaCO_3$ 0.1% and agar 1.5%. pH was adjusted to 6.7 prior to sterilisation 20 minutes at 121°C. The preculture medium was composed of glucose 0.75%, glycerol 0.75%, yeast extract (BBL) 0.135%, malt extract liquid (Wander) 0.75%, starch soluble 0.75%, *NZ*-amine Type A (Sheffield) 0.25%, soya protein 0.25%, L-asparagine 0.1%, $CaCO_3$ 0.005%, NaCl 0.005%, KH_2PO_4 0.025%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.0.01%, trace element solution 0.1%, agar 0.1%. The medium was adjusted to pH 7.0 and sterilised for 20 minutes at 121°C. The main culture medium contained glucose 2%, malt extract liquid 0.25, yeast extract (Bacto) 0.2%, soytone (Bacto) 0.2%, KH_2PO_4 0.02%, K_2HPO_4 0.04%, $MgSO_4 \cdot 7H_2O$ 0.02%, NaCl 0.005%, $CaCl_2 \cdot 6H_2O$ 0.005%, trace element solution 0.1%, agar (Bacto) 0.1%. The pH was adjusted to 6.3 before sterilisation for 20 minutes at 121°C. The trace element solution is a mixture of $FeSO_4 \cdot 7H_2O$ 0.5%, $ZnSO_4 \cdot 7H_2O$ 0.4%, $MnCl_2 \cdot 4H_2O$ 0.2%, $CuSO_4 \cdot 5H_2O$ 0.02%, $(NH_4)_6Mo_7O_{24}$ 0.02%, $CoCl_2 \cdot 6H_2O$ 0.01%, H_3BO_3 0.01%, KJ 0.005%, H_2SO_4 (95%) 0.1%.

Analytical Method

The production and the extraction-purification were monitored by TLC and HPLC.

Whole broth was combined with an equal volume of ethyl acetate. The amount of sanglifehrins was determined by thin layer chromatography (TLC) and by high performance liquid chromatography (HPLC). HPLC was performing using a LiChoCart 125-4 RP-18 column (5 μm particle size) at 50°C eluted with a triethylamine phosphoric acid (50 mM) buffer (pH 4.5) and acetonitrile (5.5 : 4.5). The flow rate was 1.5 ml per minute and the UV absorption of the eluate was monitored at 210 and 240 nm. Sanglifehrin A was eluted at a retention time of 8.6 minutes.

Cyclophilin Binding Assay

The ability of a compound to bind to cyclophilin was determined by means of a competitive binding assay (cyclophilin binding assay, CBA) as described previously[14,15]. A D-Lys^8-cyclosporin-derivative was coupled to bovine serum albumin and coated onto polyvinyl microtiter plate (1 ~ 2 μg/ml in phosphate buffered saline (PBS) for 2 hours at 37°C. After saturation of the plate with 2% BSA in PBS (1 hour at 37°C) and washings with 0.05% Tween 20 containing PBS and three times with PBS, biotinylated recombinant cyclophilin A, B or C were incubated overnight at 4°C (75 ng/ml CYP-A, 40 ng/ml CYP-B and 250 ng/ml CYP-C in 1%BSA-PBS, as titrated on BSA-CsA to achieve similar absorbance signals). After washing, the amount of bound biotinylated cyclophilin was assessed by incubation with a streptavidin coupled to alkaline phosphatase (Jackson Immunoresearch Labs, Inc, 1 : 7500 in 1%BSA-PBS, 2 hours at 37°C), followed by washing. The absorbance at 405 nm was measured after hydrolysis of *p*-nitro-phenyl phosphate (1 mg/ml in diethanolamine 1 M buffer pH 9.6, for 1 ~ 2 hours at 37°C).

In the competitive assay, biotinylated cyclophilins were incubated in the presence of the microbial extracts or compounds (overnight at 4°C). Free cyclosporin A was used as a reference compound. Solutions of CsA (1 mg/ml in ethanol) and sanglifehrinanalogues (1 mg/ml methanolic solution) were immediately added to the CyP solutions (at 1 : 10 to 1 : 100 dilution) and further 10-fold dilutions were made directly in the microtiter plate. After washings to remove the unbound CyP, the assay continued as above. Binding of a compound to the biotinylated CyP results in a decrease in the amount of CyP that can bind to the immobilised cyclosporin derivative coated on the plate and thus in a decrease in the final absorbance. The competition obtained in the presence of test compound was calculated as the percent inhibition of the control reaction between CyP and the coated cyclosporin in the absence of inhibitor. Testing serial dilutions of the microbial extracts or the test compounds allows determination of the concentration resulting in 50% inhibition of binding of the biotinylated CyP to the immobilised Cs derivative (IC_{50}). The IC_{50} for the sanglifehrins was compared with the IC_{50} for CsA run in triplicate in each microtitre plate (relative IC_{50}).

Murine Mixed Lymphocyte Reaction

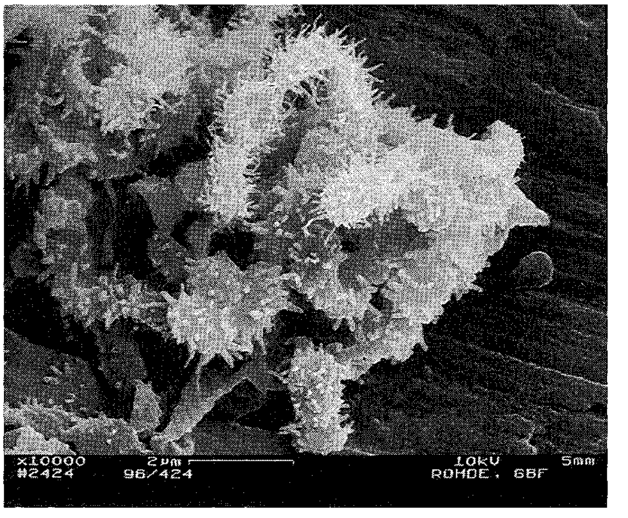
Sanglifehrins A, B, C and D and cyclosporin A were dissolved in DMSO and ethanol at 10^{-2} M and 10^{-3} M, respectively.

The mouse mixed-lymphocyte reaction (MLR) was performed according to standard procedures[16,17]. Briefly, CBA (H-2^k) and BALB/c (H-2^d) spleen cells (2×10^5 cells per well from each strain) were incubated in flat bottom tissue culture microtiter plates (Costar, Cambridge, USA) with three-fold serial dilutions of compounds (in duplicate) in 200 μl RPMI 1640 Gluta MAX I™ medium containing 10% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Gibco BLR AG, Basel, Switzerland) and 50 μM β-mercaptoethanol (Fluka Chemie AG, Buchs, Switzerland). Cell proliferation was assessed after four days by ^{3}H-thymidine incorporation. One μCi ^{3}H-thymidine (15 Ci/mmol, 1 : 1 mixture of 5 Ci/mmol and 25 Ci/mmol; Amersham, England) was added to each well. Cultures were then incubated for additional five hours, and incorporated ^{3}H-thymidine was subsequently determined according to standard procedures. The effects of compounds were quantified by subtracting the proliferation of BALB/c cells alone as background from all values. Inhibition of proliferation by compounds was calculated as percent inhibition of the proliferation of mixed cells in the absence of compounds. Concentrations required for 50% inhibition (IC_{50} values) were determined using a four parameter logistic function. Relative IC_{50} values were calculated as the ratio of the IC_{50} of the sanglifehrin analogues and the IC_{50} of cyclosporin A.

Proliferation of Murine Bone Marrow Cells

Bone marrow cells from CBA mice (2.5×10^4 cells per well) were incubated in 100 μl RPMI/10% FCS in the presence of growth factors (7.5% WEHI-3 conditioned medium and 3% L929 conditioned medium) for 4 days. ^{3}H-thymidine incorporation and IC_{50} values were determined as described above.

Fig. 1. Scanning electron microphotograph of strain A92-308110 (R. Kroppenstedt, DSM).



Results and Discussion

Taxonomy of Producing Strain A92-308110

The actinomycete strain A92-308110 was originally isolated from a soil sample collected at Dembo-Bridge (Malawi). It belongs to the genus *Streptomyces* according to the description in BERGEY's Manual, 8th edition 1974, the new edition of the BERGEY's Manual and the Prokaryotes (1992). This strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany under the accession number DSM 9954. The cell walls contain L,L-diaminopimelic acid. The fatty acids are iso- and anteiso-branched, straight and unsaturated; mycolic acids are absent. The aerial mycelium forms long chains of spores. The strain DSM 9954 grows on various organic and inorganic media and in most cases forms aerial mycelium. The primary substrate mycelium grows as hyphae and is generally beige to greyish-brown. The colour of the aerial mycelium belongs to the grey series, number 4, and this mycelium forms long chains of spores which belong to the type spira b. The spores are spiny and hairy (Fig. 1). Sclerotia are produced. Cultural characteristics of *Streptomyces* sp. A92-308110 on various descriptive media are presented in Table 1. The physiological properties of this strain are summarized in Table 2. The strain is a new *Streptomyces* designated A92-308110.

Table 1. Cultural Characteristics of strain A92-308110.

Medium pigment	Growth	Reverse	Aerial mycelium	Soluble
Glucose - asparagine agar	Moderate	Brownish	Grey	None
Glycerol - asparagine agar (ISP5)	Moderate	Brownish	Grey	None
Sucrose - nirate agar	Poor	Beige	Grey	None
Inorganic salts - starch agar (ISP4)	Moderate	Grey	Grey	None
Nutrient agar	Moderate	Beige	None	Brown
Yeast - malt extract agar (ISP 2)	Good	Grey	Grey	None
Oatmeal agar (ISP 3)	Good	Dark brown	Grey	Brown

Table 2. Physiological properties of strain A92-308110.

Temperature range for growth:	15～37°C
Nitrate reduction:	+
Tyrosine degradation:	−
Milk peptonisation	+
Hydrolysis of strach:	−
Utilizatiotn of carbon sources:	
L-Aabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	−
L-Rhamnose	−
Raffinose	−
D-Mannitol	+
Inositol	−

Production

The first isolation and characterisation of the 4 novel compounds was done from two 3000 liter tank fermentations by CypA activity guided fractionation and HPLC analysis. Agar slant cultures of the strain A92-308110 were grown for 12 days at 27°C. For the preculture 10 agar slant cultures were suspended in 100 ml of a 0.9% salt solution and two Erlenmeyer flasks of 2 liter, containing 1 liter of preculture medium were inoculated with 50 ml of this suspension. After fermentation for 24 hours at 27°C on a rotary shaker at 200 rpm with an eccentricity of 50 mm the first intermediate cultures were started.

Two 75 liter bioreactors containing 50 liter preculture medium were inoculated with 1 liter of the preculture and fermented for 96 hours at 27°C. The bioreactor was rotated at 150 rpm and air was introduced at a rate of 0.5 liter per minute per liter medium. The second intermediate culture was fermented in two 750 liter vessels each containing 500 liter preculture medium. 50 liter of the first intermediate culture were added and the fermentation was done for 70 hours at 27°C by rotating at 100 rpm and aeration of 0.8 liter per minute per liter medium. The main cultures were fermented in two 5000 liter bioreactors containing 3000 liter of the main culture medium. 300 liter of the second intermediate cultures were added to each of the vessels and grown during 96 hours at 24°C, rotating at 45 rpm and air was introduced at a rate of 0.5 liter per minute and per liter medium.

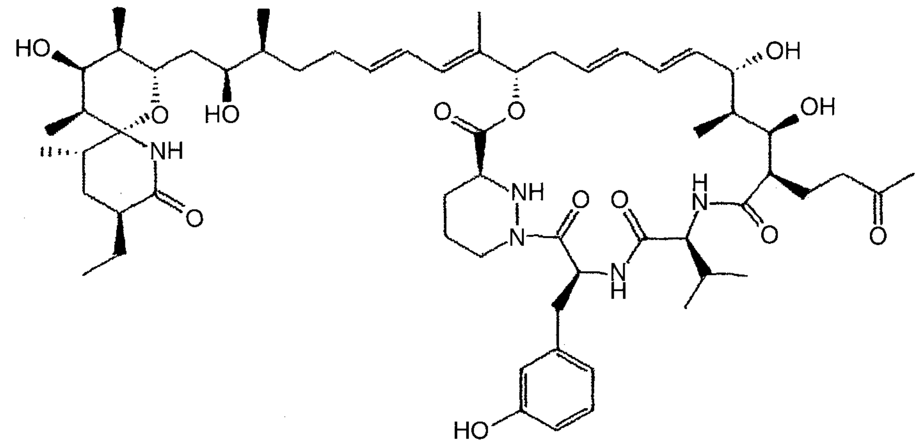
Isolation

The two 3000 liter fermentations were processed separately. 1500 liter broth were stirred with 2000 liter ethyl acetate in 4000 liter stainless steel vessel for 20 hours. The separation of the organic phase was done with a Wetsfalia-Separator type SA-20. The ethyl acetate extracts were washed twice with 80 liter of water and evaporated to dryness under reduced pressure to give 1.64 and 2 kg extracts. The two crude extracts were defatted by a three step extraction with 40 liter methanol/water 9 : 1 and 40 liter hexane. Evaporation to dryness under reduced pressure gave 1.34 kg extract.

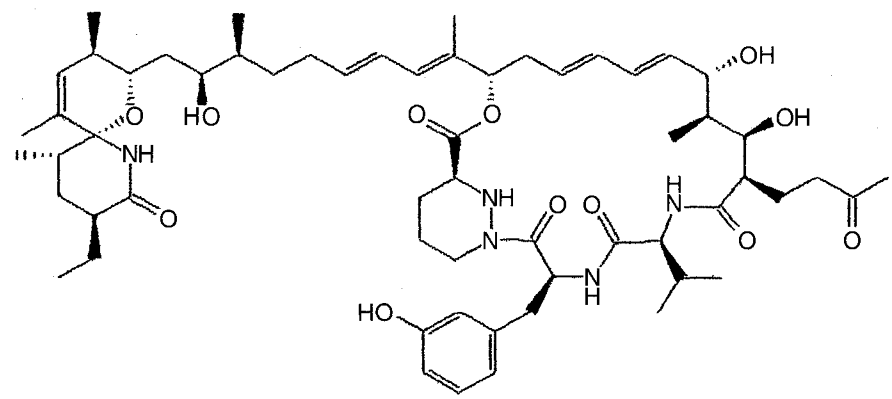
The defatted extract was chromatographed in two portions (670 g) on a column of 10 kg Sephadex H in methanol solution. Each portion was dissolved in 3.3 liters of methanol when added to the column. After collection of the first 15 liters eluate as fraction 1 the chromatography was continued by collecting 2 liter fractions. The most active fractions 2, 3 and 4 were combined to give 146 g. This sample was further chromatographed on 1 kg Silicagel Merck 0.04～

Fig. 2. Structures of sanglifehrins.

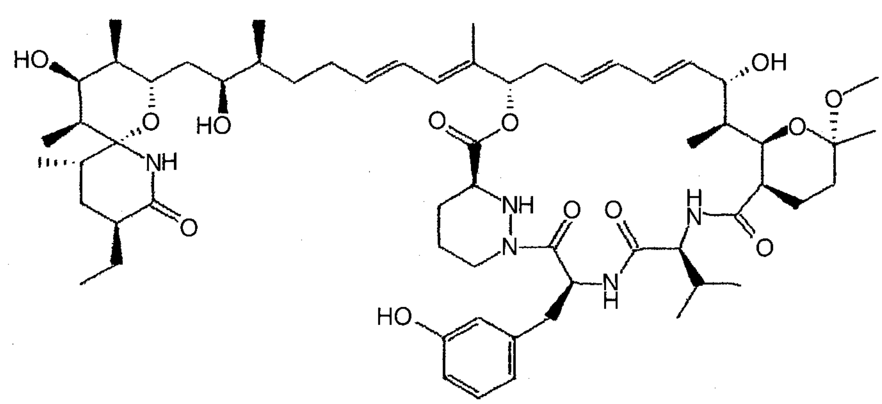
Sanglifehrin A



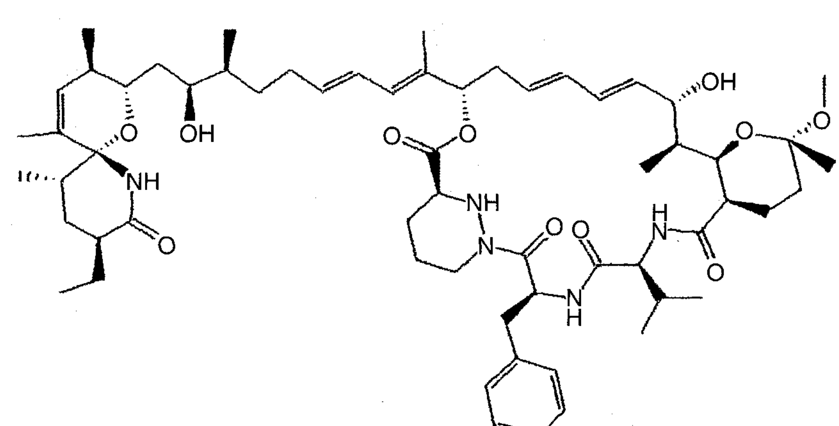
Sanglifehrin B



Sanglifehrin C



Sanglifehrin D



0.063 mm with methyl-tertiary-butyl-ether (MTBE), MTBE/5% methanol and MTBE/10% methanol. Fractions of 2 liters were collected. Fractions 5 to 9 were the most active ones and were combined to give a sample of 43.8 g. This sample was further separated on a column of 1 kg Silicagel Merck 0.04 ~ 0.063 mm with a gradient of hexane/acetone 7 : 3 to acetone. From this chromatography fraction 6 (7.0 g) was further separated on a column of 3 kg Lichroprep RP18 Merck 0.040 ~ 0.063 mm with methanol/water 94 : 6 (fraction 4 ~ 7 = 2.16 g), then on a column of 100 g Silicagel H with methylene chloride and 3% methanol (733 mg), a column of 3 kg Lichroprep RP18 with methanol/water 9 : 12 (621 mg) and than on 100 g Lichroprep RP18 with acetonitril/water 1 : 1 to yield 324 mg of pure sanglifehrin A.

Sanglifehrin B was isolated in pure form from the fractions 5 and 7 from the hexane/acetone column (7.1 g) by further chromatography on 3 kg Lichroprep RP18 0.040 ~ 0.063 mm with methanol/water 9:1 (769 mg), 100 g Silicagel H with MTBE and 3% methanol (309 mg) and finally on 100 g Silicagel H with methylene chloride and 3% methanol. The yield was 90 mg.

The fractions 9 and 10 out of the reversed phase chromatography with methanol/water 94 : 6 (7.1 g) were further purified on 100 g Silicagel H with methylene chloride/5% methanol (800 mg) and finally on 3 kg Lichroprep RP18 with methanol/water 9:1 to give 480 mg of sanglifehrin C.

The fractions 11 and 12 (835 mg) of the reversed phase chromatography with methanol/water 94 : 6 on 3 kg Lichroprep RP18 were purified on 100 g Silicagel H with MTBE/5% methanol to yield 140 mg sanglifehrin D. Based on physical and spectroscopic data (presented in the accompanying paper[18]) the compounds belong to a novel class of microbial compounds. The structures of these four sanglifehrins are given in Figure 2. The characterization of additional natural analogues is pursued. The wild strain produced in addition large amounts of mycotrienins.

Biological Properties

The relative affinity of sanglifehrins for CyPA was determined in a competitive binding assay (see Material and Methods). CsA was tested in parallel and taken as reference. The results, expressed as relative IC_{50}, which is the ratio between IC_{50} sanglifehrin and IC_{50} CsA, are shown in Table 3. The relative are shown in Table 3. Sanglifehrins A and B were shown to bind very tighly to CyPA, their affinities being twenty times higher than that of CsA (the absolute IC_{50} of Cyclosporine A in these experiments was 80 ~ 160 ng/ml). The binding affinity to cyclophilin B and C was also measured (Table 3). Sanglifehrin A was the best binder to cyclophilin A, B and C from this series of analogues, with IC_{50} of 0.05 ~ 0.09 for all three cyclophilins. Dehydration on the bicyclic spirosystem leading to sanglifehrin had little influence on the binding to CypA or CypC but it lowered the affinity to CypB approximately 6-fold. Formation of an acetal ring, giving sanglifehrins C and D resulted in a more than 10-fold decrease of binding to CyPA leading to an affinity in the same range than that of CsA. A similar decrease of binding to cyclophilin C was observed for sanglifehrins C and D and relative binding to cyclophilin B was even more drastically reduced. None sanglifehrins bound to FKBP, the binding protein of FK506 and rapamycin (data not shown).

The immunosuppressive activities of sanglifehrin A, B, C and D were assessed in two-way MLR experiments. The results are shown in Table 4 . Sanglifehrin A and B showed IC_{50} values of 170 nM and 102 nM, respectively.

Table 3. Relative IC_{50} of sanglifehrin analogues for the binding to cyclophilin A, B and C.

Compound	CYP-A	CYP-B	CYP-C
Sanglifehrin A	0.05 ± 0.02	0.09 ± 0.02	0.07 ± 0.03
Sanglifehrin B	0.05 ± 0.01	0.56 ± 0.23	0.12 ± 0.03
Sanglifehrin C	0.61 ± 0.19	2.69 ± 0.74	0.33 ± 0.01
Sanglifehrin D	0.71 ± 0.14	2.63 ± 0.62	0.62 ± 0.03

Competitive ELISA for Cyp-A, -B and -C were run in parallel. Results are expressed as the relative IC_{50} between the compound and the reference CsA tested in the same experiment and are the mean ± standard error of mean (SEM) of 2 ~ 3 independent experiments.

Table 4. Activity of sanglifehrin A, B, C and D and cyclosporin A in the murine mixed-lymphocyte reaction.

Compound	Mean IC_{50} [nM][a]	Relative IC_{50}[b]
Sanglifehrin A	170 ± 15	16
Sanglifehrin B	102 ± 7	10
Sanglifehrin C	1200 ± 104	113
Sanglifehrin D	630 ± 87	60
Cyclosporin A	10.6 ± 0.8	1

[a] Results are expressed as means ± SEM of IC_{50} values in nM; results of 3~4 independent experiments.

[b] Ratio of the IC_{50} of sanglifehrin and the IC_{50} of cyclosporin A.

Although both compounds showed a relative affinity to cyclophilin A 20-fold higher than that of CsA in the competitive cyclophilin-binding ELISA, their immunosuppressive activity in the MLR was approximately 10-fold lower. Sanglifehrin C and D were about 10-fold less active than sanglifehrin A and B with respect to both cyclophilin-binding and activity in MLR assay (IC_{50} values of 1200 nM and 630 nM, respectively). There is a correlation between the cyclophilin binding affinity of the tested sanglifehrins and the immunosuppresive activity as measured by the MLR assay. Growth factor-induced proliferation of murine bone marrow cells was not affected by sanglifehrin A at concentrations up to 5000 nM, demonstrating a specificity in the mode of action.

The sanglifehrins did not inhibit the phosphatase activity of calcineurin[3,4], the target of the cyclophilin/cyclosporin A complex[19,20]. Accordingly, they showed no activity in the reporter gene assay for IL-2 gene expression (data not shown). These results indicate that this compound class has a different mode of action than cyclosporin A. Additional studies related to the mechanism of action, to the biological effects and to selectivity of these novel microbial compounds are being pursued; from the data will be reported elsewhere.

Acknowledgments

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